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Cytoprotective effect of caffeic acid phenethyl ester (CAPE) and catechol ring-fluorinated CAPE derivatives against menadione-induced oxidative stress in human endothelial cells

Xinyu Wang,[a] Salomon Stavchansky,[a,*] Phillip D. Bowman[b] and Sean M. Kerwin[c,*]

[a]*Division of Pharmaceutics, College of Pharmacy, The University of Texas, Austin, TX 78712, USA*
[b]*US Army Institute of Surgical Research, San Antonio, TX 78234, USA*
[c]*Division of Medicinal Chemistry and Institute for Cellular and Molecular Biology, The University of Texas, Austin, TX 78712, USA*

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Abstract—Caffeic acid phenethyl ester (CAPE), a natural polyphenolic compound with many biological activities, has been shown to be protective against ischemia–reperfusion injury. We have synthesized six new catechol ring-fluorinated CAPE derivatives and evaluated their cytotoxic and cytoprotective effects against menadione-induced cytotoxicity in human umbilical vein endothelial cells. These results provide some insights into the structural basis of CAPE cytoprotection in this assay, which does not appear to be based solely on direct antioxidant properties.

1. Introduction

Caffeic acid phenethyl ester (CAPE) is a polyphenolic plant product concentrated in honeybee propolis. CAPE's reported biological properties include cancer-preventive,[1] antitumor,[2] anti-HIV,[3] immunomodulatory,[4] anti-inflammatory,[5] and antioxidant effects.[6] Recently, in animal models CAPE has been shown to ameliorate ischemia/reperfusion (I/R) injury in intestine,[7] spinal cord,[8] kidney,[9] ovary,[10] myocardia,[11] and lung.[12] In addition, acute administration of CAPE suppressed I/R-induced lipid peroxidation and injury in rat brain and kidney better than α-tocopherol.[13,14] The mechanism providing CAPE cytoprotection in I/R injury, however, has not been determined.

One of the most generally accepted mechanisms for the pathology of ischemia–reperfusion injury is the production of the reactive oxygen species (ROS) superoxide anion, hydrogen peroxide, and hydroxyl radical.[15,16] An important target of I/R injury are endothelial cells (EC). Damage to EC following I/R injury leads to delays in recovery due to poor supply of nutrients, removal of waste products, and reduction in the ability of mononuclear cell immigration to repair injured tissue. Therefore, EC provides a useful target for modeling of cytoprotectants against I/R injury in vitro. Menadione-induced oxidative stress involves the stimulated production of ROS by redox cycling and has been used as a model for evaluating the effects of oxidative stress in EC, including induction of apoptosis,[17,18] and in assessing the antioxidant activity of cytoprotectants.[19]

Despite the wide variety of biological activities associated with CAPE, only limited structural analogues have been examined,[20] especially those incorporating modifications to the caffeic acid catechol ring.[21] We are unaware of any fluorinated CAPE derivatives in the literature. Ring-fluorinated derivatives of other catechol-containing drugs may display a 'fluorine-effect,' in which the position of the fluorine ring substituent has a profound impact on the receptor binding or selectivity.[22] To better understand the structural origin of the cytoprotective effect of CAPE, we synthesized six new fluorinated CAPE derivatives. In addition to providing structure–activity information, these fluorinated CAPE derivatives may display altered metabolic lability. The introduction of fluorine on the catechol ring exerts a negative inductive effect, increases the electronic density

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* Corresponding authors. Tel.: +1 512 471 1407; fax: +1 512 471 7474 (S.S.), tel.: +1 512 471 5074; fax: +1 512 232 2606 (S.M.K.); e-mail addresses: stavchansky@mail.utexas.edu; skerwin@mail.utexas.edu

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in this conjugated system, strengthens the ester bond, and can diminish interactions with catechol methyltransferase.[23] Since the number of hydroxyl groups within CAPE catechol moiety may also play an important role in antioxidant activity,[24,25] we also examined the effect of replacing one of these hydroxyl groups with a fluorine or hydrogen, or methylating one or both catechol hydroxyl groups, on the cytoprotective activity of the resulting analogues.

2. Results

2.1. Synthesis

A Wittig coupling approach was taken to elaborate a number of fluorinated analogues of CAPE (Table 1). The required fluorinated benzaldehydes (**2a–f**) were either commercially available or easily prepared through demethylation of commercially available aldehydes with boron tribromide (Fig. 1). The demethylated aldehydes were employed directly in the subsequent Wittig reaction employing the phenethyloxycarbonylmethyl-triphenyl-phosphonium chloride **4**, which was prepared as described in the literature.[21a] In case of 2-fluoro-4,5-dimethoxybenzaldehyde **2d**, demethylation with one equivalent of boron tribromide afforded a mixture of 4- and 5-monomethylated aldehydes along with some remaining 4,5-dimethylated starting material. This mixture was used directly in the Wittig reaction to give a low yield of the monomethylated CAPE derivative **3f**,[26] along with a small amount of the 2-fluoro-dimethyl CAPE analogue **3d**. The remaining fluorinated CAPE analogues were isolated in good yield after column chromatography and recrystallization. All of the isolated CAPE analogues were characterized by ^{1}H and ^{13}C NMR and low- and high-resolution mass spectrometry. All CAPE derivatives gave satisfactory elemental analyses. In agreement with the results reported by others,[21a] the (*E*)/(*Z*) ratio of the CAPE analogues prepared in this way was generally >9:1, as judged by ^{1}H NMR.

Two series of CAPE analogues were prepared, those bearing a fluorine at the 3-position of the caffeic acid catechol ring (**3a–c**) and those with fluorine at the 2-position (**3d–f**). In addition to variation in the position of fluorination, analogues were prepared in which one (**3a, 3f**) or both (**3d**) of the hydroxyl groups of the catechol functionality of CAPE were methylated. We also examined an analogue in which one of the catechol hydroxyl groups was replaced with a fluorine (**3c**).

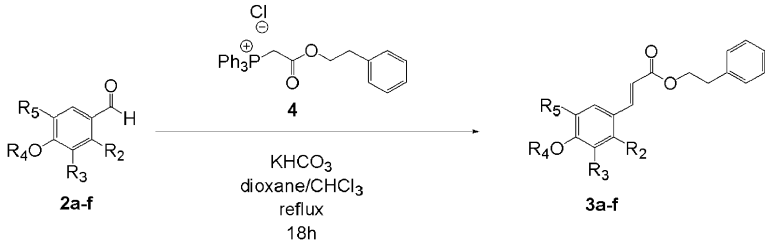
2.2. Cytotoxicity of CAPE and fluorinated CAPE analogues

CAPE and the fluorinated CAPE analogues were initially assayed for cytotoxic effects in HUVEC at the concentrations to be employed in cytoprotection studies. HUVEC were incubated in the presence of these compounds at 5, 10, and 15 μg/ml for 24 h, and the extent of cell viability was determined versus vehicle-treated control cells using the Alamar blue assay.[27] Cell viability less than 90% of control was considered toxic. The results, shown in Figure 2, demonstrate that while CAPE, **3b**, **3e**, **3a**, and **3f** were toxic at the highest concentration examined (15 μg/ml), the analogues **3d** and **3c** showed no toxicity at any tested concentrations. It was found that **3b** was less cytotoxic at 10 μg/ml than at 5 and 15 μg/ml. The origin of this phenomenon is not known; however, such non-monotonic dose–responses have been reported for other compounds[28] including caffeic acid.[29]

2.3. Cytotoprotection against menadione-induced oxidative stress

We developed an in vitro model of oxidative injury by assessing the ability of CAPE and CAPE analogues to prevent the cytotoxic effects of menadione on human umbilical vein endothelial cells (HUVEC). Menadione treatment of HUVEC for 24 h resulted in a concentration-dependent decrease in cell viability as measured by the Alamar blue assay (Fig. 3). The lowest dose of

Table 1. Synthesis of fluorinated CAPE analogues



Compound	R_2=	R_3=	R_4=	R_5=	Yield (%)
a	H	OMe	H	F	57
b	H	OH	H	F	86
c	H	H	H	F	78
d	F	H	Me	OMe	6[a]
e	F	H	H	OH	55
f	F	H	Me	OH	4[a]

[a] Low yields are due to compound losses during repeated chromatography required to separate **3d** and **3f** from the reaction product of crude **2f**, which contained some of the fully methylated **2d**.

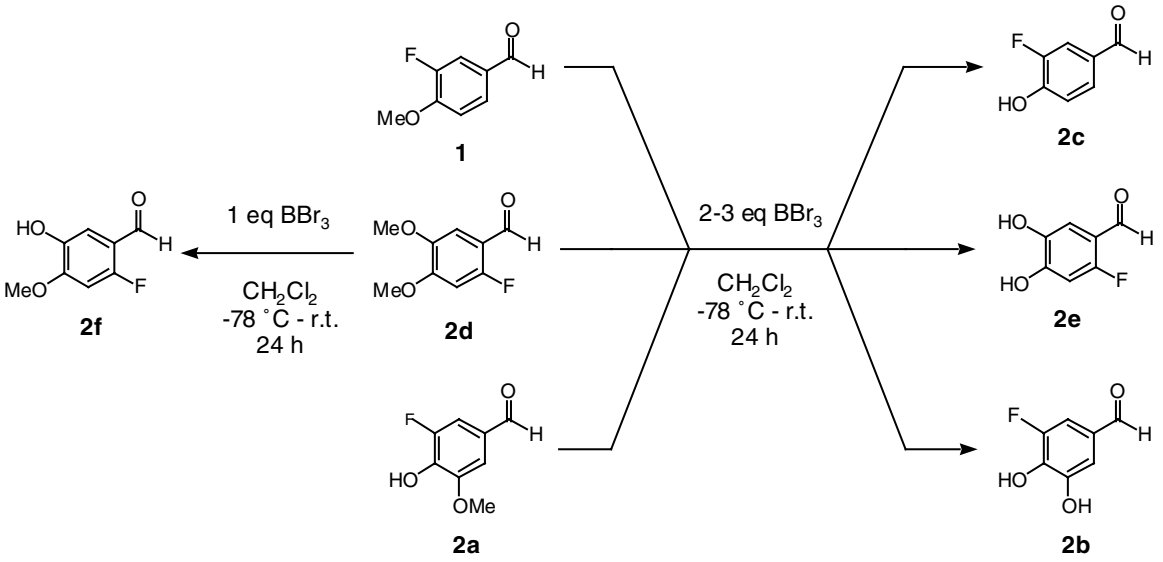


Figure 1. Synthesis of fluorinated benzaldehydes.

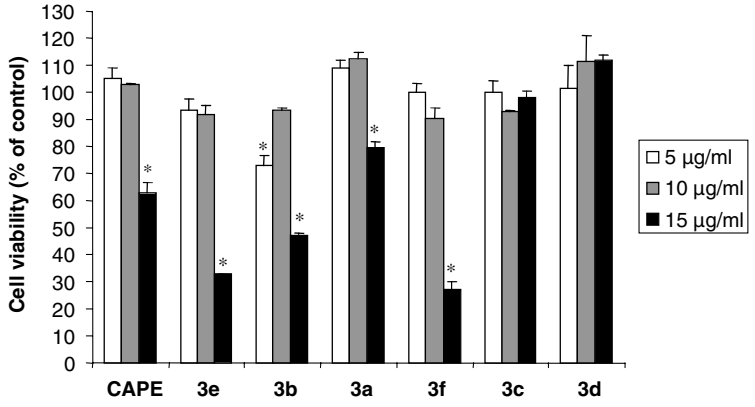


Figure 2. The cytotoxicity of CAPE and its derivatives toward HUVEC. HUVEC were incubated with CAPE at the indicated concentrations for 24 h and the number of viable cells was determined by Alamar blue assay. Values are reported as mean ($n = 3$) percent of untreated control with error bars showing the standard deviation. Asterisks indicate significant cytotoxicity relative to no treatment ($P > 0.05$).

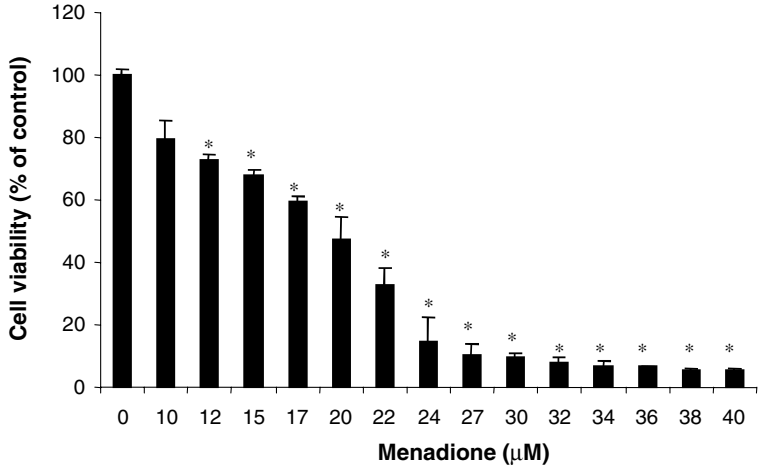


Figure 3. Menadione shows dose-dependent cytotoxicity in HUVEC. HUVEC were incubated with menadione for 24 h at the indicated concentrations for 24 h and the number of viable cells was determined by Alamar blue assay. Values are reported as mean ($n = 3$) percent of untreated control with error bars showing the standard deviation. The lowest dose of menadione causing 90% loss in cell viability (e.g., 30 µM) was employed in the cytoprotection assays. Asterisks indicate significant cytotoxicity relative to no treatment ($P > 0.05$).

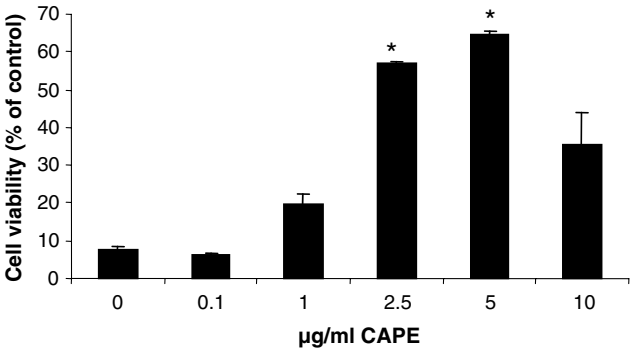


Figure 4. CAPE cytoprotection against menadione in HUVEC. Cells were pre-treated with CAPE at the indicated concentration for 6 h prior to incubation with 30 μM menadione and the remaining viable cells were determined by Alamar blue assay. Values are reported as mean ($n = 3$) percent of untreated control with error bars showing the standard deviation. CAPE at 2.5 and 5 μg/ml provided maximal cytoprotection to about 60% of untreated cells ($P < 0.001$).

menadione that reduced cell viability to about 10% of untreated cells (ca. 30 μM)[30] was employed for the cytoprotection assays of CAPE and its analogues.

Pretreatment with CAPE (0.1, 1, 2.5, and 5 μg/ml) for 6 h, prior to menadione treatment for 24 h, produced dose-dependent cytoprotection against oxidative injury (Fig. 4). The maximal protection, obtained at 5 μg/ml CAPE, resulted in >60% cell viability. At 10 μg/ml, CAPE's cytoprotective effect decreased, perhaps due to some cytotoxic effect of CAPE at this higher concentration.

The morphology of menadione-treated HUVEC is shown in Figure 5. HUVEC controls (Fig. 5A, 0.1% DMSO for 24 h) exhibit normal mitotic cells. Menadione-treated HUVEC (Fig. 5B, 30 μM menadione for 24 h) display significant shrinkage and fragmentation, morphological characteristics of apoptosis. HUVEC pretreated with 5 μg/ml CAPE 6 h before menadione treatment exhibit relatively normal cell morphology with few cells undergoing apoptosis (Fig. 5C).

Almost all CAPE derivatives showed dose-dependent cytoprotection against menadione-mediated cytotoxicity in HUVEC to some extent except for **3b**. The dose-dependent cytoprotection profiles for all of the CAPE derivatives are shown in Figure 6. Compared to CAPE, CAPE analogues **3a**, **3c**, **3d**, and **3f** display continued cytoprotective effects even at the highest concentrations, 10 or 15 μg/ml. In contrast, other structurally related antioxidants such as caffeic acid, ethyl ferulate, propyl gallate, chlorogenic acid, and rosmarinic acid did not display any cytoprotective effect in this assay at 15 μM (data not shown). Within the same passage of HUVEC, we compared CAPE and its derivatives' cytoprotection at their maximal cytoprotective dose as shown in Figure 7. CAPE analogue **3e** showed no significant difference in cytoprotection from CAPE. Compounds **3a**, **3c**, **3d**, and **3f** displayed less cytoprotective effects than CAPE, and compound **3b** had no protection for HUVEC.

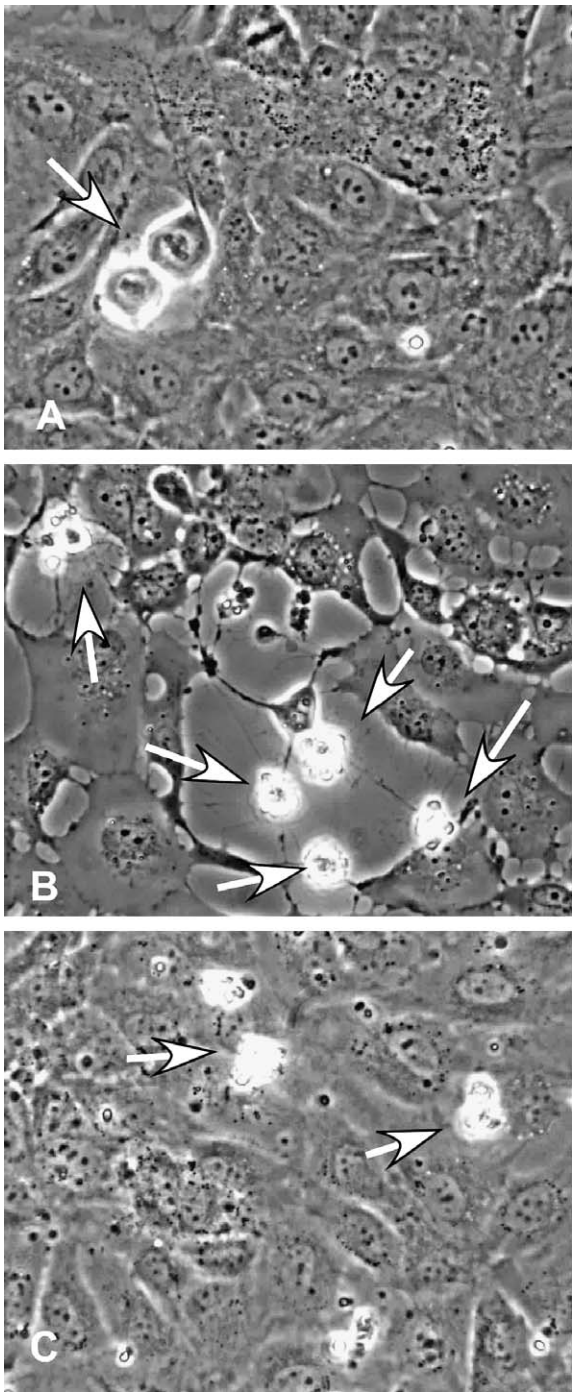


Figure 5. Morphology of HUVEC treated with 0.1% DMSO (A), 30 μM menadione alone (B), or pretreated with 5 μg/ml CAPE for 6 h before incubation with 30 μM menadione (C) for 24 h. (A) Cells undergoing normal mitosis are indicated with an arrow. (B and C) Apoptotic cells are indicated by arrows. HUVEC were protected by CAPE pretreatment against menadione toxicity, as shown by much less cell damage and fewer apoptotic cells.

3. Discussion

In accord with previous reports of CAPE's protective role in I/R injury,[7–14] we demonstrate here that CAPE is cytoprotective against menadione-induced oxidative stress in HUVEC. Pretreatment of HUVEC with CAPE protects these cells from the cytotoxic effects of menadi-

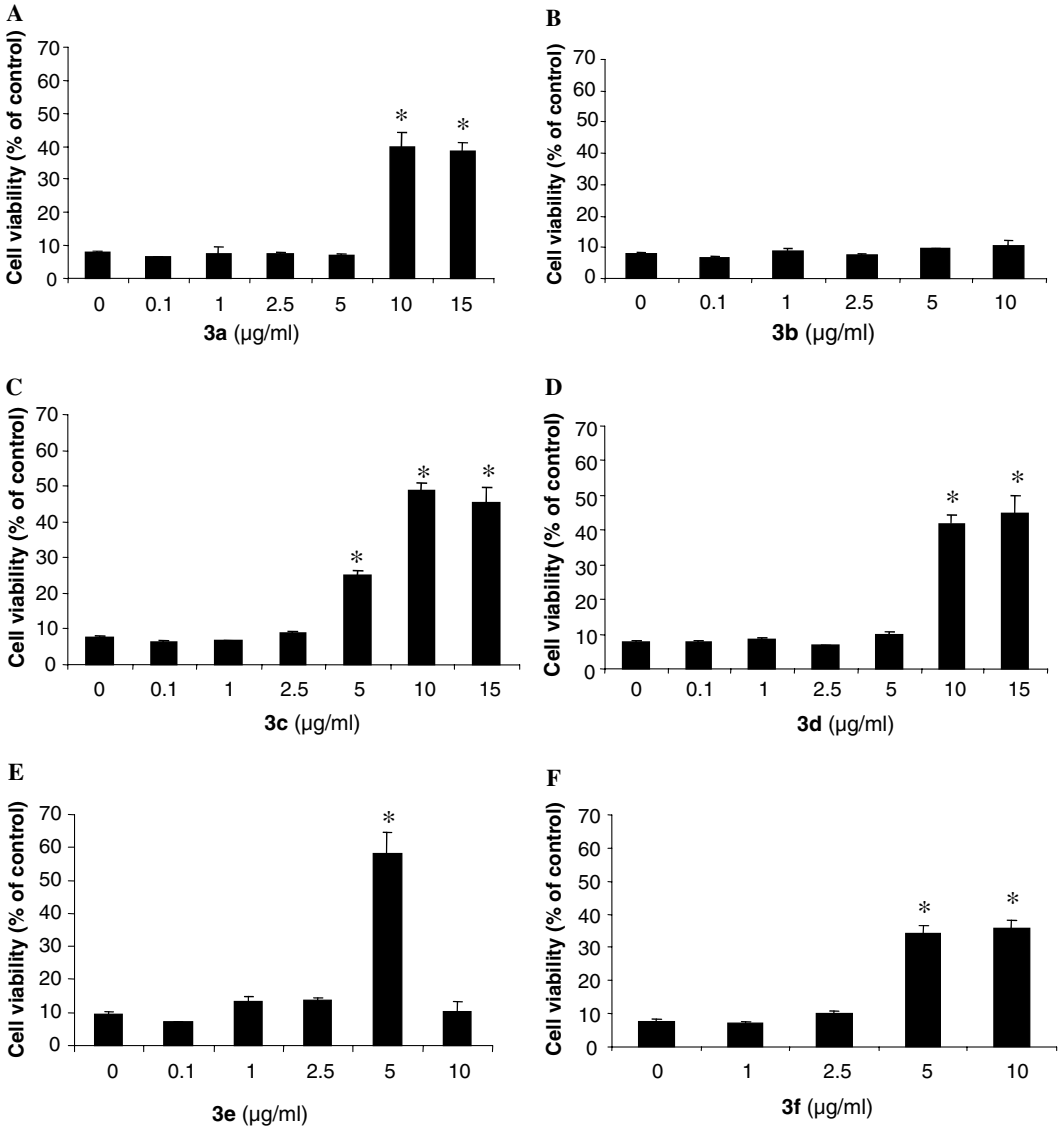


Figure 6. Dose-dependent cytoprotection by CAPE derivatives. HUVEC were incubated with CAPE analogues for 6 h at the indicated concentrations prior to menadione treatment. CAPE analogues were (A) **3a**; (B) **3b**; (C) **3c**; (D) **3d**; (E) **3e**; and (F) **3f**. Values are reported as mean ($n = 3$) percent of untreated control with error bars showing the standard deviation. Asterisks indicate significant protective effects against menadione-caused injury relative to no pretreatment ($P > 0.05$).

one. The maximal protection, at 5 μg/ml of CAPE, results in 60% cell viability compared to menadione-untreated cells. At higher concentrations of CAPE, less cytoprotection is observed, which may be related to the cytotoxicity of CAPE to HUVEC at concentrations >10 μg/ml. Interestingly, antioxidant compounds structurally related to CAPE, such as caffeic acid, failed to protect HUVEC from menadione-induced cytotoxicity. While the molecular mechanism of this cytoprotection by CAPE is not known, we note that the apoptosis of HUVEC induced by menadione treatment is also protected against by CAPE (Fig. 5).

While a variety of CAPE derivatives have been prepared,[20] relatively few analogues have been explored in which the catechol ring is substituted,[21] and no catechol-ring fluorinated CAPE derivatives have been previously reported. Previous studies of fluorinated catechols have shown that fluorine substitution at particular positions can have a profound impact on receptor binding,[23] although this is not always the case.[22b] Fluorine substitution can also diminish the susceptibility of catechols to metabolism by methyltransferases.[23c] As shown in Figure 1 and Table 1, the preparation of the fluorinated CAPE derivatives is relatively straightforward due to the commercial availability of a number of fluorinated methoxybenzaldehydes, which can be easily demethylated and immediately subjected to Wittig coupling. The methylated CAPE derivatives derived from direct Wittig coupling of these aldehydes were also prepared. With the establishment of menadione-induced oxidative stress in HUVEC as a model, we were able to study the relationship between CAPE structure, specifically catechol ring fluorination and methylation, and cytoprotection against I/R-like injury in vitro.

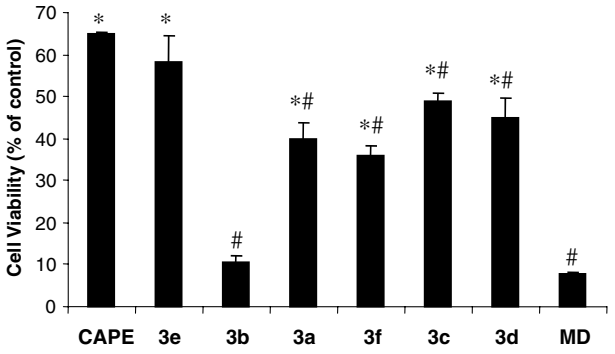


Figure 7. Comparison of CAPE and CAPE analogues' maximal cytoprotection against menadione-induced oxidative stress. CAPE: 5 μg/ml; **3e**: 5 μg/ml; **3b**: 10 μg/ml; **3a**: 10 μg/ml; **3f**: 10 μg/ml; **3c**: 10 μg/ml; **3 d**: 15 μg/ml. Values are reported as mean ($n = 3$) percent of untreated control with error bars showing the standard deviation. Asterisks indicate significant protective effects against menadione-caused injury relative to no pretreatment ($P > 0.05$). Pound signs indicate significant difference relative to CAPE pre-treated cells ($P < 0.05$).

We first note that the cytotoxicity of these CAPE analogues toward HUVEC is not correlated with the position of the fluorine substitution. The 2-fluoroCAPE derivative **3e** and the 3-fluoro CAPE derivative **3b** are cytotoxic at >10 μg/ml (Fig. 2), as is CAPE itself. In contrast, derivatives that lack CAPE's catechol functionality, such as the 2-fluoro-4,5-dimethyl analog **3d** and the fluorophenol **3c**, are less cytotoxic than CAPE at the highest concentrations examined (15 μg/ml) (Fig. 2).

The cytoprotection studies demonstrate that the position of fluorine substitution on the CAPE catechol ring can have a significant effect on the ability of these derivatives to protect against menadione-mediated oxidative stress. The presence of a fluorine at the 2-position of the catechol ring (**3e**) results in dose-dependent cytoprotection (Fig. 6e) at a level that is the same as that of CAPE (Fig. 7). In contrast, the presence of a fluorine at 3-position (**3b**) completely abolishes cytoprotective activity (Fig. 6b). The catechol functionality of CAPE and related antioxidants has been reported to be involved in cytoprotection through metal ion chelation.[31] In our studies, fluorinated CAPE derivatives in which the catechol functionality of CAPE is methylated or replaced with a phenol also display cytoprotection. Both 2- and 3-fluoromonomethyl CAPE derivatives **3f** and **3a**, respectively, retain cytoprotective activity, although the maximal cytoprotection by these analogues is less than that by CAPE. Interestingly, the 2-fluorodimethyl CAPE derivative **3d** and 3-fluoro-4-hydroxy CAPE analogue **3c** are also both cytoprotective, and these analogues, which are not cytotoxic at higher concentrations, do not show the diminution in cytoprotection at higher concentrations observed for CAPE.

4. Conclusions

The results presented here demonstrate that CAPE is cytoprotective against menadione-induced HUVEC cell death. Some of the fluorinated CAPE analogues examined here are as cytoprotective as CAPE, but a number of other naturally occurring antioxidants do not show this cytoprotective effect. While none of the fluorinated analogues examined here display cytoprotective effects greater than CAPE at similar concentrations, some less toxic and potentially more metabolically stable analogues have been identified. In addition, the cytoprotection data described here provide some insight into the structural basis for CAPE cytoprotection. CAPE is known as a potent antioxidant, and the cytoprotection that we observe against menadione-induced cell death may be related to its antioxidant activity; however, the lack of cytoprotection by other antioxidants, the observation of a pronounced effect of the position of the fluorine substituents on activity, and the lack of a structural requirement for the catechol moiety indicate that the mechanism of cytoprotection may be more complex than a direct antioxidant effect of these compounds. We are currently conducting studies to further explore the mechanism responsible for cytoprotective effects of CAPE and its derivatives.

5. Experimental

5.1. Materials and apparatus

Chemical reagents 6-fluoroveratraldehyde (**2d**), 3-fluoro-4-hydroxy-5-methoxybenzaldehyde (**2a**), 3-fluoro-4-methoxybenzaldehyde (**1**), boron tribromide, chloroacetyl chloride, phenethyl alcohol, and triphenylphosphine were purchased from Aldrich (Milwaukee, WI). Phenethyloxycarbonylmethyl-triphenyl-phosphonium chloride (**4**) was prepared from triphenylphosphine and chloroacetic acid phenethyl ester[32] as described in the literature.[21a] Menadione sodium bisulfite (menadione) and DMSO were purchased from Sigma (St. Louis, MO). All solvents were of analytical grade and distilled prior to experiments. Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} aluminum plates made from EM SCIENCE (Gibbstown, NJ). Flash chromatography was performed on silica gel column (230–400 mesh, 40–63 μm) from Fisher Scientific (Pittsburgh, PA).

Melting points were calculated from DSC-diagrams using TA Instruments Model 2920 (New Castle, DE) with a heating range of 10 °C/min and rounded to whole numbers. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 or Varian Inova 500, using CD_3OD or $CDCl_3$ as solvents. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane (TMS) as internal standard for ^{1}H NMR and in ppm relative to the solvent for ^{13}C NMR. Elemental analyses were performed on a 2400 Perkin-Elmer Elemental Analyzer by Quantitative Technologies Inc (Whitehouse, NJ). Low-resolution MS data were obtained using Finnigan MAT TSQ700 (San Jose, CA). High-resolution MS data were acquired on Micromass ZABE (Manchester, England).

6. Synthesis of CAPE derivatives

6.1. General procedure for demethylation

6.1.1. 2-Fluoro-4,5-dihydroxybenzaldehyde (2e). Compound **2d**, 2-fluoro-4,5-dimethoxybenzaldehyde (500 mg, 2.715 mmol), was dissolved in 12 ml CH_2Cl_2 and cooled to −70 °C in a dry ice/acetone bath under argon. A 1 M solution of BBr_3 in CH_2Cl_2 (8.15 ml, 8.15 mmol) was added slowly with vigorous stirring. The reaction mixture was stirred overnight without further cooling. After 24 h, MeOH (10 ml) was added to the reaction mixture to quench excess BBr_3 and the solvent was evaporated. This process was repeated two additional times and the crude product **2e** was used in the next step without further purification.

6.2. General procedure for Wittig reaction

6.2.1. 3-(2-Fluoro-4,5-dihydroxyphenyl)-acrylic acid phenethyl ester (3e). The crude 2-fluoro-4,5-dihydroxybenzaldehyde (**2e**) was dissolved in 7.5 ml of 1,4-dioxane. The phosphonium chloride **4** (1.7 g, 3.7 mmol), 7.5 ml $CHCl_3$, and $KHCO_3$ (815 mg, 8.15 mmol) were added. The reaction mixture was heated under reflux in a 110 °C oil bath with vigorous stirring under argon for 18 h. The resulting mixture was filtered, washed with CH_2Cl_2, and concentrated on a rotary evaporator. Column chromatography (EtOAc/CH_2Cl_2 (2:3, v/v)) afforded 456 mg (55% yield) of **3e** as a white solid: mp 157 °C; TLC (Silica gel, EtOAc/CH_2Cl_2 (2:3, v/v)), R_f 0.45; ^{1}H NMR (CD_3OD) δ 2.97 (t, J = 7.0 Hz, 2H), 4.35 (t, J = 7.0 Hz, 2H), 6.26 (d, J = 16.0 Hz, 1H), 6.54 (d, J = 11.7 Hz, 1H), 6.97 (d, J = 7.2 Hz, 1H), 7.23 (m, 5H), 7.66 (d, J = 16.0 Hz, 1H); ^{13}C NMR (CD_3OD) δ 36.14, 66.22, 103.96 (d, J_{C-F} = 26.4 Hz), 113.82 (d, J_{C-F} = 12.9 Hz), 114.02 (d, J_{C-F} = 3.9 Hz), 116.98 (d, J_{C-F} = 5.4 Hz), 127.52, 129.48, 129.95, 138.87 (d, J_{C-F} = 3.5 Hz), 139.35, 143.39, 150.79 (d, J_{C-F} = 11.9 Hz), 157.21 (d, J_{C-F} = 245.1 Hz), 168.90; CI-MS m/z 303 (MH^+, 100). HRCI-MS: calcd for $C_{17}H_{15}FO_4$; 303.1033. Found 303.1023; Anal. Calcd for $C_{17}H_{15}FO_4$: C, 67.54; H, 5.00; F, 6.28. Found: C, 67.53; H, 4.97; F, 6.18.

6.2.2. 3-(3-Fluoro-4-hydroxy-5-methoxyphenyl)-acrylic acid phenethyl ester (3a). The 3-fluoro-4-hydroxy-5-methoxy-benzaldehyde (**2a**, 200 mg, 1.18 mmol) was used directly in the Wittig reaction to afford 214 mg (57% yield) of compound **3a** as an off-white solid after column chromatography and recrystallization from EtOAc/hexane: mp 94 °C; ^{1}H NMR ($CDCl_3$) δ 3.02 (t, J = 7.0 Hz, 2H), 3.94 (s, 3H), 4.42 (t, J = 7.0 Hz, 2H), 6.28 (d, J = 16.1 Hz, 1H), 6.83 (d, J = 1.6 Hz, 1H), 6.95 (dd, J = 10.8, 1.8 Hz, 1H), 7.28 (m, 5H), 7.53 (d, J = 16.1 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 35.23, 56.54, 65.03, 106.03, 109.54 (d, J_{C-F} = 19.2 Hz), 117.07, 126.11 (d, J_{C-F} = 7.8 Hz), 126.60, 128.55, 128.92, 135.38 (d, J_{C-F} = 13.9 Hz), 137.88, 143.86, 148.30 (d, J_{C-F} = 6.2 Hz), 150.55 (d, J_{C-F} = 242.6 Hz), 166.77; CI-MS m/z 317 (MH^+, 100). HRCI-MS: calcd for $C_{18}H_{17}FO_4$; 317.1189. Found 317.1184; Anal. Calcd for $C_{18}H_{17}FO_4$: C, 68.35; H, 5.42; F, 6.01. Found: C, 68.24; H, 5.36; F, 5.98.

6.2.3. 3-(3-Fluoro-4,5-dihydroxyphenyl)-acrylic acid phenethyl ester (3b). Demethylation of 3-fluoro-4-hydroxy-5-methoxy-benzaldehyde (**2a**, 200 mg, 1.18 mmol) with BBr_3 (3 ml, 3 mmol) afforded crude 3-fluoro-4,5-dihydroxy-benzaldehyde (**2b**), which was used in the subsequent Wittig reaction with **4** to give 307 mg (86% yield) of **3b** as an off-white solid after column chromatography and recrystallization from EtOAc/CH_2Cl_2: mp 135 °C; ^{1}H NMR (CD_3OD) δ 2.98 (t, J = 7.0 Hz, 2H), 4.36 (t, J = 7.0 Hz, 2H), 6.25 (d, J = 15.9 Hz, 1H), 6.83 (t, J = 1.5 Hz, 1H), 6.85 (d, J = 2.0 Hz, 1H), 6.87 (d, J = 2.0 Hz, 1H), 7.24 (m, 5H), 7.45 (d, J = 15.9 Hz, 1H); ^{13}C NMR (CD_3OD) δ 36.18, 66.22, 108.36 (d, J_{C-F} = 19.8 Hz), 111.84, 116.75, 126.62 (d, J_{C-F} = 8.9 Hz), 127.55, 129.51, 129.97, 137.36, 139.41, 145.92, 148.84 (d, J_{C-F} = 5.9 Hz), 153.43 (d, J_{C-F} = 238.1 Hz), 168.76; CI-MS m/z 303 (MH^+, 100). HRCI-MS: calcd for $C_{17}H_{15}FO_4$; 303.1033. Found 303.1030; Anal. Calcd for $C_{17}H_{15}FO_4 + 1/4H_2O$: C, 66.55; H, 5.09. Found: C, 66.76; H, 4.84.

6.2.4. 3-(3-Fluoro-4-hydroxyphenyl)-acrylic acid phenethyl ester (3c). Demethylation of 3-fluoro-4-methoxy-benzaldehyde (**1**, 200 mg, 1.30 mmol) with BBr_3 (3.3 ml, 3.3 mmol) afforded crude 3-fluoro-4-hydroxy-benzaldehyde (**2c**), which was used in the subsequent Wittig reaction with **4** to give 290.6 mg (78% yield) of compound **3c** as an off-white solid after column chromatography and recrystallization from EtOAc/hexane: mp 80 °C; ^{1}H NMR ($CDCl_3$) δ 3.01 (t, J = 7.1 Hz, 2H), 4.42 (t, J = 7.1 Hz, 2H), 6.27 (d, J = 16.0 Hz, 1H), 6.98 (s, 1H), 7.00 (s, 1H), 7.01 (s, 1H), 7.26 (m, 5H), 7.55 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 35.22, (40.48), 65.10, 114.72 (d, J_{C-F} = 18.5 Hz), 116.83, 117.71, 125.57 (d, J_{C-F} = 3.1 Hz), 126.60, 127.67 (d, J_{C-F} = 6.3 Hz), 128.54, 128.94, 137.87, 143.66, 145.79 (d, J_{C-F} = 14.7 Hz), 151.16 (d, J_{C-F} = 238.9 Hz), 167.04; CI-MS m/z 287 (MH^+, 100). HRCI-MS: calcd for $C_{17}H_{15}FO_3$; 287.1083. Found 287.1089; Anal. Calcd for $C_{17}H_{15}FO_3 + 1/4H_2O$: C, 70.21; H, 5.37; F, 6.53. Found: C, 70.02; H, 5.27; F, 6.50.

6.2.5. 3-(2-Fluoro-4,5-dimethoxyphenyl)-acrylic acid phenethyl ester (3d). This compound was a co-product from the above reaction leading to compound **3f**. It was isolated as 69 mg (6% yield) of an off-white solid after repeated column chromatography and recrystallization from CH_2Cl_2/hexane: mp 86 °C; ^{1}H NMR ($CDCl_3$) δ (ppm): 3.02 (t, J = 7.1 Hz, 2H), 3.88 (s, 3H), 3.89 (s, 3H), 4.43 (t, J = 7.1 Hz, 2H), 6.36 (d, J = 16.1 Hz, 1H), 6.64 (d, J = 11.6 Hz, 1H), 6.94 (d, J = 6.8 Hz, 1H), 7.26 (m, 5H), 7.78 (d, J = 16.1 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 35.24, 56.28, 56.40, 64.99, 100.15 (d, J_{C-F} = 28.0 Hz), 109.42 (d, J_{C-F} = 4.5 Hz), 113.46 (d, J_{C-F} = 12.7 Hz), 117.64 (d, J_{C-F} = 6.0 Hz), 126.57, 128.53, 128.94, 137.17 (d, J_{C-F} = 3.0 Hz), 137.92, 145.65 (d, J_{C-F} = 2.5 Hz), 152.01 (d, J_{C-F} = 10.2 Hz), 156.51 (d, J_{C-F} = 248.8 Hz), 166.95; CI-MS m/z 331 (MH^+, 100). HRCI-MS: calcd for $C_{19}H_{19}FO_4$; 331.1346. Found 331.1354; Anal. Calcd for $C_{19}H_{19}FO_4 + 1/4H_2O$: C, 68.15; H, 5.87. Found: C, 68.47; H, 5.70.

6.2.6. 3-(2-Fluoro-4-methoxy-5-hydroxyphenyl)-acrylic acid phenethyl ester (3f). Partial demethylation of 2-fluoro-4,5-dimethoxy-benzaldehyde (**2d**, 600 mg, 3.26 mmol) was carried out with one equivalent of boron tribromide (3.26 ml, 3.26 mmol) to give crude monomethyl-2-fluoro-4,5-dihydroxybenzaldehyde (**2f**) as a mixture of 4- and 5-methyl isomers containing some unreacted **2d**. This mixture was used directly in the subsequent Wittig reaction with **4** to afford 42 mg (4% yield) of compound **3f** an of off-white solid after repeated column chromatography and recrystallization from CH_2Cl_2/hexanes: ^1H NMR ($CDCl_3$) δ 3.01 (t, $J = 7.1$ Hz, 2H), 3.89 (s, 3H), 4.41 (t, $J = 7.1$ Hz, 2H), 6.33 (d, $J = 16.1$ Hz, 1H), 6.61 (d, $J = 11.3$ Hz, 1H), 7.05 (d, $J = 7.1$ Hz, 1H), 7.26 (m, 5H), 7.74 (d, $J = 16.1$ Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 35.23, 56.30, 65.02, 99.45 (d, $J_{C-F} = 28.5$ Hz), 112.38 (d, $J_{C-F} = 4.0$ Hz), 114.52 (d, $J_{C-F} = 13.2$ Hz), 118.02 (d, $J_{C-F} = 6.2$ Hz), 126.56, 128.52, 128.96, 137.04 (d, $J_{C-F} = 3.1$ Hz), 137.93, 142.00 (d, $J_{C-F} = 2.5$ Hz), 149.01 (d, $J_{C-F} = 10.2$ Hz), 155.76 (d, $J_{C-F} = 247.8$ Hz), 167.01; CI-MS *m/z* 317 (MH^+, 100). HRCI-MS: calcd for $C_{18}H_{17}FO_4$; 317.1189. Found 317.1198; Anal. Calcd for $C_{18}H_{17}FO_4$: C, 68.35; H, 5.42. Found: C, 68.04; H, 5.22.

6.3. Cell culture

Human umbilical vein endothelial cells (Cascade Biologics, Portland, OR) were cultivated on 1% gelatin-coated 75-cm^2 culture flasks (Corning Incorporated, Corning, NY) in Medium 200 supplemented with 2% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), and Fungizone (0.25 μg/ml) supplied by Cascade Biologics. The cells were cultivated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with medium changes every 2 days until confluent. Prior to an experiment, HUVEC were subcultivated with Trypsin/EDTA onto gelatin-coated 48-well multiplates (Corning Incorporated, Corning, NY) at 5000 cells/cm^2, grown to confluence, and kept for 72 h to produce a quiescent cell layer closely resembling an endothelium in vivo. On the day before the experiment, the medium was changed. Only the second through fifth passage of the cells were used.

6.4. Cell viability (Alamar blue assay)

Stock solutions of menadione (0.5 M in PBS) or CAPE analogues (1000× in DMSO) were diluted in culture medium and added to plate wells at final concentrations of 10–40 μM and cell viability was assessed at 24 h after initiation of treatment versus vehicle (PBS or DMSO) controls using Alamar Blue™ (Biosource International, Camarillo, CA), which is converted to a fluorescing compound in amounts proportional to the number of viable cells.[22] The cells were incubated for 2 h at 37 °C with culture medium containing 10% Alamar Blue™. After incubation, fluorescence was measured at 545 nm excitation and 590 nm emissions using SpectraMAX® M2 microplate reader (Molecular Devices, Sunnyvale, CA). HUVEC were also regularly observed under phase contrast microscopy and images were captured with a digital camera.

6.5. In vitro cytoprotection assay

Confluent HUVEC were pretreated with CAPE and its derivatives for 6 h. Cytotoxic dose of menadione was then added to the HUVEC in the presence of testing compounds and DMSO as vehicle control. After 24 h incubation, cell viability was measured using the Alamar blue assay.

6.6. Statistical analysis

Data were represented as means ± standard deviation. Differences among the groups were analyzed using the one-way analysis of variance combined with Tukey (equal variances assumed) or Games–Howell (equal variances not assumed) test. A difference of $p < 0.05$ was considered significant for all statistical analyses.

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